AMENDMENTS TO THE SPECIFICATION:

Please replace the paragraph spanning page 15, line 27 to page 16, line 3, with the following amended paragraph:

The cysteine variants of the present invention can be used for any of the known therapeutic uses of the native proteins in essentially the same forms and doses all well known in the art. By way of example, therapeutic methods for increasing hematopoeisis in a patient and for accelerating recovery from neutropenia are described herein which use a cysteine variant of granulocyte macrophage colony stimulating factor (GM-CSF) according to the present invention. Also described ~~herin~~ herein are therapeutic methods for accelerating recovery from neutropenia in patients using cysteine variants of granulocyte colony stimulating factor (G-CSF), therapeutic methods for increasing red blood cell formation and increasing hematocrit levels in patients using a cysteine variant of erythopoietin (EPO), and therapeutic methods for inhibiting growth of cancer cells in patients using cysteine variants of alpha interferon. It is to be understood, however, that general discussion regarding modes of administration, dosage and delivery of cysteine variants such as the GM-CSF, G-CSF, EPO and alpha interferon cysteine muteins, is generally intended to apply to therapeutic methods using any of the cysteine variants described herein.

Please replace the paragraph on page 28, lines 31-37, with the following amended paragraph:

In accordance with the present invention, a suitable single dose size is a dose that results in the desired therapeutic effect in the patient, depending ~~o~~ on the cysteine mutein that is administered, or in the amelioration of at least one symptom of a condition in the patient, when administered one or more times over a suitable time period. Doses can vary depending upon the disease being treated. One of skill in the art can readily determine appropriate single dose sizes for a given patient based on the size of a patient and the route of administration.

Please replace the paragraph on page 57, lines 9-19, with the following amended paragraph:

DNA sequences encoding alpha interferon-2 can be amplified from human genomic DNA, since alpha interferon genes do not contain introns (Pestka et al., 1987). The DNA sequence of alpha interferon-2 is given in Goeddel et al. (1980). Alternatively, a cDNA for alpha interferon-2 can be isolated from human lymphoblastoid cell lines that are known to express alpha interferon spontaneously or after exposure to viruses (Goeddell et al., 1980; Pickering et al., 1980). Many of these cell lines are available from the American Type Culture Collection (~~Rockville, MD~~10801 University Blvd., Manassas, VA 20110-2209). Specific mutations can be introduced into the alpha interferon sequence using plasmid-based site-directed mutagenesis kits (e.g., Quick-Change Mutagenesis Kit, Stratagene, Inc.), phage mutagenesis strategies or employing PCR mutagenesis as described for GH.

On page 94, immediately following line 15, please add the following paragraphs, subheadings and Tables:

Example 35

Refolding and Purification of IFN-α2 Cysteine Muteins

Methods for expressing, purifying and determining the in vitro and in vivo biological activity of recombinant human alpha interferon 2 (IFN-α2) and IFN-α2 cysteine muteins are described in PCT/US00/00931. Methods for constructing cysteine muteins of IFN-α2 and preferred sites within the IFN-α2 protein for the locations of added cysteine residues also are described in PCT/US98/14497 and PCT/US00/00931. The following muteins have been constructed in E coli using those methods: C1S, Q5C, 43C44, N45C, Q46C, F47C, Q48C, A50C, D77C, C98S, Q101C, T106C, E107C, T108C, S163C, E165C, *166C, D2C, L3C, T6C, S8C, T52C, G102C, V103C, G104C, V105C, P109C, L11OC, M111C, S160C, L161C, R162C and K164C. One preferred method for expressing IFN-α2 in E. coli is to secrete the protein into the periplasm using the STII leader sequence. A fraction of the secreted IFN-α2 is soluble and can be purified by column chromatography as described in PCT/US00/00931. Certain cysteine muteins of IFN-α2 remain insoluble when secreted into the E. coli periplasm using the STII leader sequence. SDS-PAGE analysis of the osmotic shock supernatants of the muteins showed most to have reduced (as compared to wild type) levels of the 19 kDa rIFN-α2 band. SDS-PAGE analyses of whole cell lysates and the insoluble material from the osmotic shocks revealed that

these muteins were expressed at relatively high levels but accumulated primarily in an insoluble form, presumably in the periplasm. These proteins comigrated with wild type rIFN-α2 standards under reducing conditions indicating that the STII leader had been removed. Qualitative assessments of relative expression levels of the muteins are summarized in Table 17. Procedures for refolding insoluble, secreted IFN-α2 proteins have not been described previously. The following protocol (here referred to as "Protocol I") was developed to express and refold IFN-α2 cysteine muteins into a biologically active form.

For expression of IFN-α2 cysteine muteins and IFN-α2, typically, a 325 ml culture in a 2 liter shake flask, or a 500 ml culture in a 2 liter baffled shake flask, were grown at 37°C in a gyrotory shaker water bath at ~170-220 rpm. Cultures were grown, induced, harvested, and subjected to osmotic shock as described in PCT/US00/00931. Resulting supernatants and pellets were processed immediately or stored at –80°C.

IFN-α2 cysteine muteins that were recovered as insoluble proteins in the osmotic shock pellets were denatured, reduced and refolded into their proper conformations using the following refold procedure. The pellet from the osmotic shock lysate was first treated with B-PER™ bacterial protein extraction reagent as described by the manufacturer (Pierce). B-PER is a mild detergent mixture that disrupts the *E. coli* membranes and releases the cytoplasmic contents of the cells. Insoluble material was recovered by centrifugation, resuspended in water, and recentrifuged. The resulting pellet was solubilized in 5 mL of 6 M guanidine, 50 mM cysteine in 20 mM Tris Base. The mixture was allowed to stir for 30 minutes before being dialyzed overnight at 4°C against 400 mL of 40mM sodium phosphate, 150 mM NaCl, pH 8.0. The next day the pH of the refold mixture was adjusted to 3.0 and the mixture was centrifuged before being loaded onto an S-Sepharose column, followed by a Cu^{++} IMAC column as described for the purification of rIFN-α2 from the osmotic shock supernatant in PCT/US00/00931. Six IFN-α2 cysteine muteins: Q5C, C98S, Q101C, T106C, E107C and *166C have been refolded and purified using these procedures. Similar procedures can be used to refold and purify insoluble wild type IFN-α2.

Non-reducing SDS-PAGE analysis of purified Q5C, C98S, Q101C, T106C, E107C, and *166C cysteine muteins showed that the muteins were recovered predominantly as monomers, migrating at the expected molecular weight of ~ 19 kDa. C98S migrated with a slightly higher

molecular weight than the other rINF-α2 muteins due to the absence of the native Cys1-Cys-98 disulfide bond. Some of the purified muteins contained small amounts of disulfide-linked rIFN-α2 dimers. The molecular weights of the dimer species were approximately 37-38 kDa.

When processing a number of cyteine muteins of IFN-α2, it was discovered that certain cysteine muteins appeared to be present in both the soluble and insoluble fractions following cell lysis. Ratios of soluble verus insoluble IFN-α2 protein varied from mutant to mutant. Therefore, an alternative solubilzation/refolding procedure (here referred to as "Protocol II") that involves a whole cell solublization step was developed to enhance recovery of the IFN-α cysteine muteins. A modification of the culture methods was found to improve the efficiency of processing of the STII leader sequence and was employed to express IFN-α cysteine muteins for refolding and purification, as detailed below. In the modified method, 325-400 ml cultures were grown in LB media containing 100 mM MES, pH 5.0 and 100 μg/ml ampicillin at 37°C with vigorous shaking, e.g., 220-250 rpm in a New Brunswick C25KC environmental shaker, to a cell density of 0.5-0.7 OD at 600 nm. Cultures were then induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.5 mM and upon induction the temperature was reduced to 28°C and the shaker speed was reduced to 140 rpm. Induced cultures were incubated overnight (14-18 hours) and harvested by centrifugation. Cell pellets were processed immediately or stored at –20°C or -80°C until processing. The cell pellets derived from a 325-400 mL induced culture are first suspended in 10 mL of 8 M Guanidine, 20 mM Cysteine, 20 mM Mes, 2% Tween 20, pH 3 and mixed until a homogeneous suspension is present. The pH is then increased to between pH 8-9 and the solubilization mixture is stirred for 3 hours. The cell lysate is next diluted 1:20 with ice cold renaturation buffer (20 mM Tris, pH 0.3 M guanidine, 1 M urea, 40 μm copper sulfate, pH 8). The cloudy suspension is allowed to sit 1-2 days at 4°C. The refold is clarified by centrifugation followd by a pH adjustment to 3 and second round of centrifugation. The supernatant is diluted 1:4 with cold water and load onto a 5 mL S-Seph Hi Trap. The ion exchange column is eluted with a 100 mL gradient of 0-70% Buffer B, with Buffer A being 20 mM Mes, pH 5 and Buffer B being 10% Ethylene glycol 500 mM NaCl, 20 mM Mes pH 5. Alternatively, refolded IFN-α cysteine muteins can be captured from the refold mixture using a HIC column, such as a Phenyl-Sepharose column. The refold mixture is first centrifuged, ammonium sulfate is added to the supernatant to a final concentration of 10%, the

mixture is recentrifuged, and the supernatant loaded onto a 10 mL Phenyl Sepharose column equilibrated in 10% ammonium sulfate, 20 mM Tris, pH8. IFN-α cysteine muteins are eluted from the column using a 100 mL linear gradient from 10% ammonium sulfate, 20 mM Tris pH 8 to 30% ethylene glycol, 20 mM Tris, pH 8. The interferon pool from a Phenyl-Sepharose column can be further purified using a copper chelating column, S-Sepharose column or both.

Interferon cysteine muteins also can be solubilized and refolded using other reducing agents that also act as cysteine blocking agents. Substitution of reduced glutathione, thioglycolic acid or cysteamine for cysteine in the solubilization/refold mixtures yielded refolded, soluble IFN cysteine variants that could be purified and PEGylated following the procedures described in **Example 37**. When no reducing agent or 20 mM DTT was substituted for cysteine in the solubilization/refold mixtures, yields of refolded, soluble IFN cysteine muteins were reduced to non-detectable levels when the refold mixture was analyzed by Reversed Phase HPLC. Additionally, no refolded, soluble IFN cysteine mutein was recovered following S-Sepharose chromatography of the refold mixture when no reducing agent or 20 mM DTT was substituted for cysteine in the solubilization/refold mixtures.

The following muteins were expressed in *E coli*, refolded and purified using Protocol II: C1S, Q5C, 43C44, N45C, F47C, Q48C, A50C, C98S, Q101C, T106C, E107C, S163C, E165C, *166C, D2C, L3C, T6C, S8C, T52C, G102C, V103C, G104C, V105C, P109C, L11OC, M111C, S160C, L161C, R162C and K164C. These refolds were performed at pH 8 or in some instances 7.5.

Example 36

Bioactivities of IFN-α2 Cysteine Muteins

Biological activities of the purified Q5C, C98S, Q101C, T106C, E107C, and *166C IFN-α2 cysteine muteins that were purified using Protocol I of **Example 35** were measured in the Daudi growth inhibition assay described in PCT/US00/00931. Protein concentrations were determined using Bradford or BCA protein assay kits (Bio-Rad Laboratories and Pierce). Commercial wild type rIFN-α2 and rIFN-α2 prepared as described in PCT/US00/00931 were analyzed in parallel on the same days to control for interday variability in the assays. The muteins inhibited proliferation of Daudi cells to the same extent as the wild type rIFN-α2 control proteins, within the error of the assay. Mean IC_{50}s for five of the muteins (Q5C, Q101C, T106C,

E107C and *166C) were similar to the mean IC_{50}s of the wild type rIFN-α proteins, ranging from 15-18 pg/ml. The mean IC_{50} for the C98S protein was 28 pg/ml. These data are summarized in Table 17.

Table 17.

Expression and *in vitro* Bioactivities of IFN-α2 Cysteine Muteins

IFN-α2 Protein	Mutation Location	Relative Expression		Form Assayed	Mean IC_{50} (pg/ml)	IC_{50} Range[3] (pg/ml)
		Total Cellular [1]	Percent Soluble [2]			
rIFN-α2 [4]	-	-	-		16 +/- 7	8-29 (n=10)
rIFN-α2 [5]	-	++++	~ 33	Soluble	13 +/- 4	7-19 (n=10)
C1S	N-terminal region[6]	+/-	0			
Q5C	N-terminal region	++++	~ 20	Refolded	17	15, 17, 20
43C44	A-B loop	++	0			
N45C	A-B loop	++	0			
Q46C	A-B loop	+/-	0			
F47C	A-B loop	++++	~5			
Q48C	A-B loop	+/-	0			
A50C	A-B loop	+/-	0			
D77C	B-C loop	+/-	0			
C98S	C-helix [7]	+++++	~ 5-10	Refolded	28	22,30,32
Q101C	C-D loop	+++++	~ 5-10	Refolded	18	10,22,23
T106C	C-D loop	+++++	~ 5-10	Refolded	18	18,18
E107C	C-D loop	+++++	~ 5-10	Refolded	18	8,22,24
T108C	C-D loop	+/-	0			
S163C	C-terminal region	++++	~ 33			
E165C	C-terminal region	+++	~ 20			
*166C	C-terminus	+++	~ 20	Refolded	15	8,16,20

[1] Relative accumulation of the IFN-α2 protein in whole cell extracts
[2] Portion of the IFN-α2 protein in the osmotic shock supernatant, determined from SDS-PAGE gels
[3] IC_{50} values from individual experiments. A range is shown when N > 5.
[4] Commercial wild type rIFN-α2 (Endogen, Inc.)
[5] Wild type rIFN-α2 prepared by Bolder BioTechnology, Inc.
[6] Mutation creates a free cysteine (C98) in the C-helix
[7] Mutation creates a free cysteine (C1) in the N-terminal region

Biological activities of the following muteins, purified using Protocol II of **Example 35,** were measured in the Daudi growth inhibition assay described in PCT/US00/00931: C1S, D2C, L3C, S8C, N45C, F47C, C98S, V103C, V105C, E107C, M111C, R162C, S163C, K164C, E165C and *166C. The observed IC_{50}s are listed in Table 18 along with IC_{50}s for wild type rIFN-α protein controls used in the same experiments.

Table 18.

In vitro Bioactivities of IFN-α2 Cysteine Muteins Purified by Protocol II with and without PEGylation

IFNα2 Mutant	Mutation location	IC_{50} [1] (pg/ml)	IC_{50} (pg/ml), 20K PEG-Protein [1]
RIFN-α2 [2]	-	15 to 55	-
RIFN-α2 [3]	-	16 to 109	-
C1S[4]	N-terminal region	120, 130	100, 160
D2C	N-terminal region	39	300
L3C	N-terminal region	24, 75	105, 270
S8C	N-terminal region	37	220
N45C	A-B loop	52	104
F47C	A-B loop	66, 56, 58	120, 72, 240
C98S[5]	C-helix	105, 110,100	500, 720, 900
G104C	C-D loop	110	600
V105C	C-D Loop	38	33
E107C	C-D loop	90, 98, 110	160, 220, 180
M111C	C-D Loop	40	190
R162C	C-terminal region	600	4000
S163C	C-terminal region	70,50, 88	310, 125,360
K164C	C-ter	100	600
E165C	C-ter	43, 60, 51	160, 220, 300
*166C	C-terminus	48, 78 , 96	120, 300

[1] IC_{50} values from individual experiments. A range is shown when N > 5.
[2] Commercial wild type rIFN-α2 (Endogen, Inc.)
[3] Wild type rIFN-α2 prepared by Bolder BioTechnology, Inc.
[4] Mutation creates a free cysteine (C98) in the C-helix
[5] Mutation creates a free cysteine (C1) in the N-terminal region